REMARKS

Claims 86-107 are pending upon entry of the above recited amendments. Claims 86, 93, 94, 104 and 107 are independent claims.

Claims 44-49, 51-53, 63, 70-79, 83 and 84 are cancelled in this Amendment. Claims 1-43, 50, 54-62, 64-69, 81 and 82 were cancelled in prior amendments. Applicant has considered, but does not agree, with the reasons stated in the Office Action for rejecting claims 44-49, 51-53, 63, 70-79, 83 and 84. However, in the interest of advancing prosecution of this application, Applicant has submitted new claims, and for the reasons set forth below respectfully submits that the claims are in acceptable form for allowance.

Applicant respectfully submits that by declaration or otherwise Applicant is not attempting to confuse the record or improperly broaden the scope of the invention. Applicant is attempting in good faith to advance the prosecution of this application. The new claims correlate generally, but not identically, to prior claims as indicated in the chart below. As explained below, the new claims are fully supported by the application as filed and do not include any new matter. Entry of the amendments is respectfully solicited.

OLD	NEW	OLD	NEW	OLD	NEW	OLD	NEW	OLD	NEW
44	86	49	91	71	96	76	97	84	106
45	87	52	93	72	98	77	102	85	107
46	88	53	92	73	99	78	103		
47	89	63	94	74	100	79	104		
48	90	70	95	75	101	83	105		

The present invention is based on the concept that neuronal progenitor cells are transiently treated by exposure to a differentiation-promoting factor ("intermittent treatment" to prime the cells at page 5, lines 2-4), resulting in the treated cells being considered "determined" to differentiate completely or predominantly into a specific cell type (page 2, lines 3-9). The factor is removed after a period of time (page 5, lines 10-13). After removal of the differentiation-promoting factor, the treated, determined cells preferably are expanded, that is, proliferate so that many more will be available for use as needed. Since the differentiation-promoting factor is removed during expansion, the treated, determined cells do not further differentiate. The treated, determined cells differentiate into dopaminergic neurons only after a further stimulus by a subsequent treatment (page 2, lines 6-9). The treated, determined cells maintain their ability to mitose (page 3, lines 17-19), such that they may be subcloned and/or expanded (page 5, lines 10-14).

Thus, according to the invention, there are three types or stages of cells. The first stage includes pluripotent neuronal progenitor cells, which are treated by transient exposure to the differentiation-promoting factor to produce the second stage cells. The second stage cells are treated, determined cells. When the treated, determined cells are subsequently treated with an additional contact with the differentiation-promoting factor, typically in vitro, or by transplantation, typically in vivo, they will differentiate into dopaminergic neurons, the third stage, at which time, the cells are no longer mitotic.

The neuronal tissue claimed in this application consists essentially of the treated, determined cells prior to the subsequent treatment. This neuronal tissue then serves a source of cells that differentiate into substantially only dopaminergic neurons upon transplantation or additional contact with a differentiation-promoting factor for a time sufficient to allow such complete differentiation.

With this background into the presently amended claims, issues raised in the Office Action will now be addressed.

Rejection under 37 CFR § 1.75(c)

Former claim 52, which correlates to pending claim 93, has been objected to as being in improper dependent form for failing to limit the subject matter of the claim upon which it depends. Applicant has further amended the correlating claim 93 such that it is independent and thus this objection is moot. Withdrawal of this objection is respectfully solicited.

Rejections Under 35 U.S.C. § 112 First Paragraph

The Office Action states that former claims 44 and 63, which correlate to pending independent claims 86 and 94, respectively, improperly list differentiation-promoting factors that apply to all neurons. These independent claims are directed to neuronal tissue consisting of treated, determined cells that will differentiate into substantially only dopaminergic cell types, upon a certain claimed subsequent treatment of the treated, determined cell. Accordingly, the claims now recite that the differentiation-promoting factor is selected from the group consisting of glial cell line-derived neurotrophic factor, leukemia inhibitory factor, interleukin-1, interleukin-11, interleukin-1b, and thyroid hormone. As noted in the Office Action at pages 3-4, the specification discloses the above-referenced list of factors as those that will result in differentiation into dopaminergic cell types.

The Office Action also states that the specification's disclosure that a high percentage of the cells of the tissue should be specific neurons does not support recitation in the claims of "neuron progenitors." Pending claims 87, 88, and 95 have been amended in light of this objection, and are supported at least at page 2, lines 15-18, of the specification, in light of lines 5-11 of the same page. Ultimately, once the tissue is first treated, it contains cells that were once progenitors, but post treatment are highly determined. The first treatment is discussed at page 5 of the specification as priming.

After the subsequent treatment of the treated, determined cells, they will differentiate into a high percentage of dopaminergic cell types (e.g. dopaminergic neurons). The specification at page 2, lines 6-9, discloses, "these cells become determined to differentiate completely or predominately into a specific cell type (e.g. dopaminergic neuron) after transplantation or, in

general, after making contact with an appropriate substrate." This references aspect (2) of the subsequent treatment as claimed. Support for aspect (1) of the subsequent treatment is at page 5, lines 12-13, providing: "Said primed progenitor cells respond to a subsequent treatment with such factors more readily." Support for the subsequent treatment can also be found at least at page 3, lines 24-25.

The terms "partial differentiation" or "partially differentiated" have been completely removed from the claims, thus objections to this term, specifically in reference to repeating step (d) of claim 94 as recited in claim 96, is moot. Applicant respectfully submits that step (d), "treating the progenitor cells by transiently exposing the progenitor cells to the differentiation-promoting factor, wherein the treated cells become determined;" is supported at least at page 5, lines 1-16, of the specification. This paragraph discloses priming with a compound that promotes specific differentiation, and that priming may be repeated several times.

The Office Action also asserts that there is no proper antecedent basis for recitation of certain steps under sub-atmospheric oxygen levels, or simulations thereof. Applicant respectfully disagrees. Applicant has disclosed performance of steps (c), (d), and (e) of current claim 94 under reduced oxygen levels in the specification at least at the following: page 3, lines 26-29, "[e]xpansion of neuronal progenitor cells may include a **variation of atmospheric oxygen content**, priming, transient or non-transient expression of foreign genes, treatment of exogenous compounds, especially under **reduced oxygen partial pressure**...," and lines 31-33 of the same page, "selection of determined progenitor cells includes generation of clonal cell lines, which may include a variation, **especially a reduction, of atmospheric oxygen;**" page 4, lines 2-12, including, "[p]roliferation of selected cells may be promoted using a **modulation of atmospheric oxygen content...each under reduced oxygen content of the atmosphere**, "and lines 20-30 of the same page. Further support can be found at page 9, lines 14-16, "priming with exogenous factors that stimulate differentiation, expression of foreign genes and/or **changes in atmospheric oxygen and nitrogen concentrations**," and page 10, lines 16-27. Furthermore, original claim 14 recites, "partial differentiation of progenitor cells may be performed in **conditions with reduced or increased oxygen/nitrogen content**...," and original claim 23

recites, "proliferation of progenitor cells is achieved **under hypoxic conditions**...," which also support the newly submitted claims.

Hypoxia is a condition of low oxygen compared to normal levels. Partial pressure is defined as the fraction of the total pressure due to each constituent of a gas mixture. The partial pressures are proportional to the concentrations of the individual gases in a mixture. HACKH'S CHEMICAL DICTIONARY 490 (4th ed. 1969). Thus, Applicant has disclosed performance of steps (c), (d) and (e) under sub-atmospheric levels. Even though the claim language itself is not identical to all the instances of the concept in the original application, the meaning is understood in the art to be equivalent. Whether the step is performed under hypoxic conditions, reduced oxygen partial pressure, or reduced oxygen content, these all refer to sub-atmospheric oxygen levels.

Similarly, performance of steps (c), (d) and (e) under simulated reduced atmospheric oxygen content is supported in the application at page 4, lines 8-11, "[t]he effect induced by the reduction of sub-atmospheric oxygen content may be simulated or enhanced using conditions that exert similar effects on cell metabolism...." Specific reference is made to at least proliferation at page 4, line 2, and subcloning at page 4, line 6, under reduced oxygen or simulations thereof. Additional support for proliferation under simulated reduced oxygen content is disclosed at lines 30-31 of page 4 of the specification, "[a]s mentioned above similar effects may be obtained using inhibitors of mitochondrial respiration."

The Office Action also objects to former claim 83, which correlates to pending claim 105, in that it recites a marker but does not recite a transfected cell. Applicant agrees. "Marker" in claim 105 refers to the term in the biological, and not necessarily transgenic, sense. Applicant respectfully submits that for the purpose of claim 105, the term "marker" is in reference to a measurable characteristic which a person of skill would recognize is indicative of a state, disease, or in this case cell type. For example, a "cell surface marker" is any molecule characteristic of the plasma membrane of a cell or in some cases of a specific cell type. Support for this claim can be found at least at page 9, lines 6-8, and page 12, lines 9-10 of the application.

The Office Action additionally objects to what correlates to claim 94, step (f) and claim 104, clause (b) on the basis that the claims as previously presented included new matter, but does not specify precisely what is objected to in these claim sections. Nonetheless, Applicant has amended these correlating claims and respectfully submits they are fully supported in the application.

Rejections Under 35 U.S.C. § 112 Second Paragraph

Applicant respectfully submits that the newly added claims do not refer to a universal differentiating-promoting factor. Also, the term "synthetic" has been removed from the claims. Although Applicant does not agree that the term "partially differentiated" are indefinite in the now cancelled claims, Applicant has deleted the terms from in the instant claims, rendering this objection moot.

The Office Action states that the claims are contradictory because neurons, which are fully differentiated, are not actively dividing. Yet the claims were characterized by the Office Action as directed to neurons with the capacity to divide indefinitely. The Office Action states that only by undergoing de-differentiation would a neuron become mitotic, and in the process lose its specific neuron characteristics. This point is crucial to the invention and touches upon the complete novelty of this application. By undergoing the procedure herein disclosed, actively dividing neuronal cells that *do* exhibit biological markers typical in dopaminergic neurons *are* devised such that they retain mitotic ability without losing the markers. Because the terms "partial differentiation" and "partially differentiated" have been removed from the claims in an effort to advance prosecution, Applicant submits that the pending claims avoid the alleged contradiction and that the language of the current claims comports with the requirements of patentability. Furthermore, the claims are not directed to neurons, but neuronal tissue consisting of neuronal progenitor cells that have been treated by transient contact with the differentiation-promoting factor.

In light of the claim amendments and above remarks, Applicant respectfully requests reconsideration and withdrawal of all 35 U.S.C. § 112 objections.

Rejections Under 35 U.S.C. § 102

Applicant agrees that claim 1 of the Boss reference claims a culture of neuronal progenitor cells. However, the instant invention is readily distinguishable over Boss, and Applicant disagrees that Boss teaches or even suggests the invention as claimed. Furthermore, Applicant objects to the assertion that Boss clearly teaches partially differentiated neuronal progenitor cells, considering that "partially differentiated" has been characterized in the Office Action as indefinite. Therefore, Applicant again traverses the rejection over Boss.

It is not only material, but also crucial that the cells of the tissue of instant invention are mitotic because this feature allows for continuous cell division that is required for repeated subcloning, i.e. potentially unlimited production of the tissue. Boss claims cells that can divide *at most* eight times, severely limiting the potential for sub-cloning and continued life of the cultures.

Additionally, the instant claims are for tissue made of neuronal progenitor cells, *but only such cells that have undergone the disclosed treatment* resulting in a physiological change in the cells. No longer are these cells progenitors poised for differentiation into one of many types of neurons or glial cells. Rather, after the first treatment they are highly determined, and after the subsequent treatment they will differentiate substantially into dopaminergic cell types (e.g. dopaminergic neurons). This is a significant distinction because (1) glial cells can invoke an immune response if implanted in a host, and (2) neuronal cells that respond to signals other than dopamine (e.g. GABAergic or cholinergic cells) may be used to treat different conditions depending on the signal. Boss claims a culture of neuron progenitor cells that *are* poised for differentiation, that *may* develop into one of many types of neurons or glial cells. Therefore, the instant claims are clearly distinguishable over Boss.

The claims are also distinguishable over Luskin. Luskin claims progenitor cells isolated from the anterior subventrical zone (SVZa) of the mammalian brain, only. This is stated in Luskin at page 7, lines 21-24, page 9, lines 14-15, "[t]he cells comprising the herein described

composition can be isolated from the SVAa of the brain of any mammal of interest," and page 11, lines 10-11. This zone was targeted in Luskin, because as stated at page 2, lines 24-25, the SVZa is "known to be a source of certain dividing cells in the nervous system." The instant claims begin with cells from the midbrain region. The midbrain, also referred to as the mesencephalon, connects the pons Varolii (part of the hind brain) with the interbrain and hemispheres. GRAYS ANATOMY 652 (15th ed. 1995). In contrast, the SVZa is just below the lateral ventricles of the brain. The lateral ventricles are within the cerebral hemisphere. GRAYS ANATOMY at 639. Thus, the SVZa and the midbrain regions are different regions.

Enclosed is a very recently published paper as independent evidence that the current claims of this application are distinguishable over the prior art, as the paper describes something presented as new, yet simultaneously describes tissue very similar to what Applicant claims. *See Xuan* Wang *et al*, *Distinct efficacy of pre-differentiated versus intact fetal mesensephalon-derived human neural progenitor cells in alleviating rat model of Parkinson's disease*, Int. J. Devl. Neuroscience 22 (2004) 175-183, a copy of which is attached to this Amendment. *Wang* treats neuronal progenitor cells with a differentiation-promoting factor to induce differentiation into dopaminergic cells. *Wang*, at 176. These cultured, determined, cells proliferated for up to six months. *Id* at 178. Importantly, *Wang* notes the difficulty in the art with cultivation of cells from regions of the brain other than the SVZ. *Wang* at 181. Furthermore, *Wang* shows that treatment of progenitor cells to set them on a particular differentiation path without losing full mitotic ability, yet exhibiting some characteristics of dopaminergic neurons, *is* possible. *Wang* at 181-82. This is very similar to what Applicant has discovered and claimed, only Applicant did so many years prior to publication of *Wang*.

Applicant respectfully requests reconsideration and withdrawal of the prior art rejections.

CONCLUSION

It is respectfully submitted that claims 86-107 are distinguished over the cited art and are fully compliant with 35 U.S.C. § 112. Applicant requests reconsideration and withdrawal of all the objections and rejections. Early allowance of all of the claims pending after entry of this amendment is earnestly solicited.

Please direct all communications to the undersigned attorney, since Kristyne Bullock, the former attorney handling this application, is no longer with Akin Gump Strauss Hauer & Feld LLP.

Respectfully submitted,

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Distinct efficacy of pre-differentiated versus intact fetal mesencephalon-derived human neural progenitor cells in alleviating rat model of Parkinson's disease

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Abstract

Neural progenitor cells have shown the effectiveness in the treatment of Parkinson's disease, but the therapeutic efficacy remains variable. One of important factors that determine the efficacy is the necessity of pre-differentiation of progenitor cells into dopaminergic neurons before transplantation. This study therefore investigated the therapeutic efficacy of mesencephalon-derived human neural progenitor cells with or without the pre-differentiation in alleviating a rat model of Parkinson's disease. We found that a combination of 50 ng/ml fibroblast growth factor 8, 10 ng/ml glial cell line-derived neurotrophic factor and 10 μM forskolin facilitated the differentiation of human fetal mesencephalic progenitor cells into dopaminergic neurons in vitro. More importantly, after transplanted into the striatum of parkinsonian rats, only pre-differentiated grafts resulted in an elevated production of dopamine in the transplanted site and the amelioration of behavioral impairments of the parkinsonian rats. Unlike pre-differentiated progenitors, grafted intact progenitors rarely differentiated into dopaminergic neurons in vivo and emigrated actively away from the transplanted site.

These data demonstrates the importance of pre-differentiation of human progenitor cells before transplantation in enhancing therapeutic potency for Parkinson's disease.

Keywords: Neural progenitor cells; Parkinson's disease; Cell differentiation; Cell transplantation; Migration; Dopaminergic neurons; Central nervous system

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic (DAergic) neurons in the midbrain and, consequently, depletion of dopamine (DA) in the striatum of the patients (Olanow and Tatton, 1999). Intracerebral transplantation of human fetal mesencephalon tissue or cells, aimed at the replacement of degenerated DAergic neurons as well as the replenishment of DA neurotransmitter, has been proved a promising approach for treating PD (Lindvall et al., 1990; Freed et al., 1992; Olanow et al., 1996). The wide application of these therapies, though, has been hindered by the difficulty of obtaining adequate amounts of embryonic tissue. Particularly, the results from the clinical trials recently was unsatisfied for elder patients of PD (Freed et al., 2001). Since neural progenitor cells (NPCs) have the capacity of both self-renewal and multiple-differentiation (McKay, 1997; Gage, 2000), they might be an appropriate alternative source of cells for clinical application and offer a promising future for cell replacement therapies.

Abbreviations: PD, Parkinson's disease; DA, dopamine; NPC, neural progenitor cell; IL, interleukin; LIF, leukemia inhibitory factor; GDNF, glial cell line-derived neurotrophic factor; FGF, fibroblast growth factor; 6-OHDA, 6-hydroxydopamine; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; bFGF, basic FGF; FITC, fluorescein isothiocyanate; TH, tyrosine hydroxylase; ir, immunoreactivity; DOPAC, dihydroxyphenylacetic acid; GFAP, glail fibrillary acidic protein; CLSM, confocal laser scanning microscopy; SVZ, subventricular zone

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Up to now, extensive studies have been documented on the PD treatment with transplantation of NPCs derived from experimental animals. In addition, a few studies have been reported about therapeutic effects of transplantation of NPCs from human sources as human NPCs are considered to be a better source than animals in the clinical use. In 1997, Svendsen and co-workers observed some reliable alleviation of the rat PD model with transplantation of human whole brain NPCs (Svendsen et al., 1997), however, the effect of mesencephalon-derived human NPCs was studied seldom. Our hypothesis is that mesencephalic NPCs might possess more advantages than other brain areas during treating PD, because some researches have showed that, compared with those from striatum and cortex, mesencephalon-derived NPCs more readily differentiated into DAergic neurons after transplantation (Svendsen et al., 1996; Ostenfeld et al., 2002). In addition, what recently has been focused on more and more is that whether the pre-differentiation into DAergic neurons should be a key step before transplantation. While the studies conducted so far demonstrate the usefulness of NPC transplantation in treating PD, results from either rodent or primate animal models remain inconsistent. It is possible that the inconsistent observations from different laboratories may be due to the engrafted NPCs being obtained from different regions or species among various experiments. It has been reported that most mouse NPCs preferentially differentiated into DAergic neurons when transplanted into the striatum of parkinsonian rat (Yang et al., 2002); but another group reported that none of engrafted rat NPCs became DAergic neurons in the striatum unless cells had been treated with cytokines in vitro before transplantation (Carvey et al., 2001). The latter studies showed that pre-differentiated NPCs by a cocktail cytokines including interleukin (IL)-1, IL-11, leukemia inhibitory factor (LIF) and glial cell line-derived neurotrophic factor (GDNF) in vitro functioned on the behavioral improvement of parkinsonian rats relative to intact NPCs after transplantation (Carvey et al., 2001). Thus, pre-differentiated tissues seem to have different biological properties from undifferentiated tissues after transplantation. But the differential therapeutic efficacy of mesencephalon-derived human NPCs with or without pre-differentiation has not been documented.

In the present study, we used a combination of fibroblast growth factor (FGF) 8, GDNF and forskolin to induce the differentiation of human fetal mesencephalic NPCs into DAergic neurons in vitro. We proposed that this pre-differentiation influence their roles relative to intact NPCs, and thereby result in a differential therapeutic efficacy between the two cell populations after transplanted into the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats. In addition, the different behaviors of two kinds of grafts in vivo were investigated in order to find out the possible differential mechanisms. Our aim was to provide an appropriate cell transplantation method for clinical PD therapy.

2. Experimental procedure

2.1. Cultivation and differentiation of mesencephalon-derived human NPCs

Human NPCs were isolated from the ventral mesencephalon of a natural aborted human fetus at 12-weeks gestational age. Permission to use human embryonic tissue was granted by the Ethical Committee of Peking University. The procedure of obtaining NPCs was as described previously (Vescovi et al., 1999). Briefly, the tissue was dissected, washed with cold D-Hank's solution and minced thoroughly. Tissue fragments were mechanically separated into single cells and were cultivated in serum-free neurosphere-forming medium containing Dulbecco's modified Eagle's medium (DMEM):F12 (1:1), B27 supplements (1:50, Gibco), 20 ng/ml epidermal growth factor (EGF, Gibco), 20 ng/ml basic fibroblast growth factor (bFGF, Gibco) and 10 ng/ml LIF (Chemicon). Neurospheres were passaged every 2 weeks. After four passages, NPCs were identified by double-staining the neural stem cell specific protein-nestin and the proliferation-related nuclei antigen-Ki67. NPCs as neurospheres were fixed with 4% paraformaldehyde solution, and immersed in 30% sucrose overnight. After embedding in OCT and quick refrigeration, neurospheres were sliced by cryoultramicrotome at 10 μm and mounted on the poly-L-lysine-coated slices. After blocked with 10% normal goat serum, slices were incubated with mouse anti-Ki67 (1:100, BD) and rabbit anti-nestin (1:200, Chemicon) primary antibodies, and then with fluorescein isothiocyanate (FITC)- and rhodamine-conjugated secondary antibodies (1:200, BD). The staining lack of the primary antibody incubation was used as the negative control.

For DAergic differentiation, NPCs at the four passage were plated onto the poly-L-lysine-coated glass coverslips for immunostaining or seeded in the culture plates for Western blot analysis. After EGF, bFGF and LIF withdrew, a mixture of 50 ng/ml FGF8 (PeproTech EC Ltd., London, England), 10 ng/ml GDNF (Gibco) and 10 μM forskolin (Sigma) was added to the culture and incubated for 7 days followed by testing the expression of tyrosine hydroxylase (TH), which is the first and rate-limiting enzyme in the procedure of DA synthesis. Cells on coverslices were fixed by 4% paraformaldehyde, blocked with 10% normal goat serum and incubated with mouse anti-TH (1:1000, Sigma) primary antibody and FITC-conjugated secondary antibody. Images were examined under fluorescence microscope. Intact NPCs and the NPCs cultured without any induction factor were used as the controls. To assess the percentage of TH-immunoreactivity (ir) cells, induced cultures on three coverslips were counted in five randomly selected microscopic visual fields per coverslip.

For Western blot analysis, cells were washed twice with 0.1 M PBS and lysed with 200 μl lysis buffer (0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, 0.5% deoxycholic

acid, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF and 1% NP-40). Equal amounts of protein were loaded under reducing conditions onto a 10% SDS gel. After electrophoresis, the protein was blotted onto a polyvinylidene difluoride membrane. The membrane was blocked by skimmed milk and incubated with anti-TH antibody (1:2000, Sigma). Signals were visualized by incubating with horse radish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagent (NEN™ Life Science Products), then photographed using Kodak 1D image analysis software. Normal rat striatum, intact NPCs and NPCs cultured without any induction factor were used as the controls.

2.2. Generation of parkinsonian rat model

Total 50 adult female Sprague–Dawley rats (8 weeks of age, 250–350 g) were maintained in a 12-h light/dark cycle in cages with free access to food and water. All surgical interventions and animal care were provided in accordance with the Guide for the Care and Use of Laboratory Animal Models of the NIH. The hemi-parkinsonian rats were generated by unilateral injection of 4 μl of 6-OHDA (Sigma, 2 μg/μl dissolved in 0.9% saline containing 0.2 mg/ml ascorbic acid) into the right medial forebrain bundle at the following coordinates: AP = −4.3 mm, ML = −1.5 mm, DV = −7.5 mm (Paxinos and Watson, 1996). The injection rate was at 1 μl/min and the needle was left in place for an additional 5 min before being slowly retracted. Rats were given an amphetamine (Sigma, 5 mg/kg injected intraperitoneally) challenge 4 weeks later and their rotational scores were collected for 60 min in a computer-assisted rotometer system (Rota-count 8, Columbus instruments, Columbus, OH). Only rats exhibiting six or more net ipsilateral rotations/min were selected for further study.

2.3. Cell transplantation and behavioral test

Parkinsonian rats (successful models, $n = 36$) were randomly classified into three groups transplanted respectively with basic medium (negative control), intact NPCs and pre-differentiated NPCs by the combination of FGF8, GDNF and forskolin for 7 days in vitro. Cells for transplantation were dispersed and suspended in basic DMEM/F12 (1:1) media with no any cytokine, and were counted by typan blue exclusion with viability above 95%. Live cells for transplantation were set at a density of 100,000/μl (Fitoussi et al., 1998). The cell suspension was injected into two sites of DA-depleted striatum of rats (2.5 μl for each site). The coordinates were set as AP = +1.0 mm, ML = −3.0 mm, DV= −4.1/−5.0 mm (Paxinos and Watson, 1996). All animals were immunosuppressed with cyclosporin A (10 mg/kg/day, injected intraperitoneally, Novartis, Pharma AG, Basel, Switzerland,) 24 h before transplantation and then throughout the entire survival period. Behavioral improvements were tested by amphetamine-induced rotation examination as described before at 4, 8 and 12 weeks after transplantation, respectively.

2.4. Measurement of DA production

Rats were sacrificed at 12 weeks after transplantation by decapitation. The striatum was immediately collected, weighed, homogenized in 0.1 M perchloric acid and centrifuged. DA and its metabolite dihydroxyphenylacetic acid (DOPAC) were determined by HPLC with electrochemical detection using a reverse phase column. Results were presented as the ratio of the content of lesioned (right) side versus that of contralateral (left) side.

2.5. Assessment of cell transplantation

Rats were deeply anesthetized with an overdose of chloral hydrate at 12 weeks after transplantation. Intracardiac perfusion was performed with 0.1 M PBS followed by 4% paraformaldehyde. Brain tissues were dissected, post-fixed and cryoprotected as described above. Fixed brains were sliced on a freezing microtome (30 μm) and the serial sections at 180 μm intervals throughout the graft were collected for further processing. Sections were pretreated with 0.3% H_2O_2 to quench endogenous peroxidase, blocked with 3% normal horse serum, and then incubated respectively with mouse anti-human nuclei (1:200, Chemicon) and mouse anti-TH (1:2000, Sigma) primary antibodies overnight at 4 °C. Then they were incubated with rat-absorbed biotinylated horse anti-mouse secondary antibody (1:200, Vector Laboratories). After incubation with peroxidase conjugated avidin-biotin complex (1:100, Vector Laboratories), signals were developed by exposure to 0.02% 3,3′-diaminobenzidine and 0.03% H_2O_2. The survival and migration of grafted human cells were assessed by immunostaining of human nuclei-ir cells on the brain section and that out of the striatum. DAergic neurons in striatum were estimated by counting the number of TH-ir cells in the graft sites of each animal at 180-μm intervals through the graft. For immunofluorescent staining, incubation with H_2O_2 was eliminated. Sections were incubated with rabbit anti-neurofilament-M (1:200, Chemicon) for neuronal phenotype and rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, NewMarkers) for glial phenotype, double-stained with human nuclei antibody respectively, and then with corresponding fluorescence conjugated secondary antibodies. Antigen colocalization was verified by analyzing a series of sections at 2 μm thick throughout the specimens under the confocal laser scanning microscopy (CLSM, Leica, Heideberg, Germany). The percentage of double-positive cells in the striatum was estimated roughly by counting the cell number in five randomly selected microscopic visual fields per section. The contralateral (nontransplantation) side was used as the control relative to the ipsilateral (transplantation) side.

2.6. Statistical analysis

All data were expressed as the mean ± S.E.M. Student's *t*-test was used to compare the ratio of cytokine-converted TH-ir neurons with control in the differentiation study in vitro, and to compare the difference of the number of TH-ir cells differentiated from grafted pre-differentiated and intact NPCs in vivo. Behavioral recovery in animals and DA and DOPAC contents were analyzed using a one-way analysis of variance (ANOVA SPSS software), as appropriate, followed by a Bonferroni (Dunn) comparison of groups using least squares-adjusted means. Probability levels of <0.05 were considered statistically significant.

3. Results

3.1. Proliferation and differentiation of NPCs in vitro

Human fetal mesencephalic cells began to form primary neurospheres, an aggregation of NPCs, after 2 weeks of cultivation (Fig. 1A), and continued to form more secondary and tertiary neurospheres after each passage in vitro for up to 6 months. In our experiment, cultivation and neurosphere formation of NPCs derived from the human mesencephalon were relatively difficult, as compared to those derived from the other brain areas such as the subventricular zone (SVZ) and hippocampus, or rodent animal brains (data not shown). But, once neurosphere formed, the proliferation and survival of NPCs derived from this area had no difference as compared with those obtained from the other brain areas. Most cells in the neurospheres co-expressed nestin and Ki67 (Fig. 1B and C), suggesting that they remained in a primordial state and held self-renewal capacity at the same time. Intact NPCs as neuronspheres did not express TH (data not shown). After the mitogens (EGF, bFGF and LIF) were removed from the culture media, cells migrated from the neurospheres and began to differentiate, but seldom converted into TH-ir cells spontaneously (Fig. 1D). When incubated with a combination of 50 ng/ml FGF8, 10 ng/ml GDNF and 10 μM forskolin for 7 days, NPCs were induced to become TH-ir cells (26.19 ± 2.45%, Fig. 1E), a significant increase relative to the controls (1.40 ± 0.53%, $P < 0.01$). The apparent up-regulation of TH expression by the cytokine mixture was confirmed using Western blot analysis (Fig. 1F).

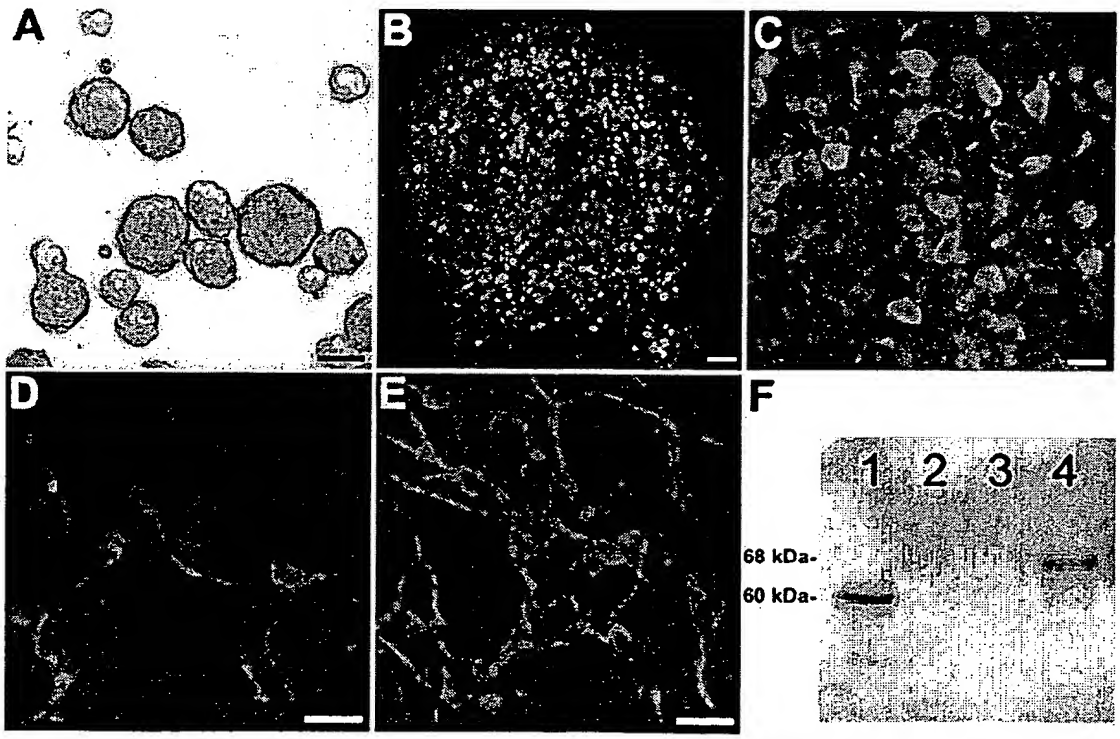

Fig. 1. Proliferation and differentiation of human fetal mesencephalic NPCs in vitro. Human mesencephalic NPCs proliferated as floating neurospheres when cultured in a serum-free medium supplemented with EGF, bFGF and LIF (A). Most cells in the neurospheres coexpressed nestin (red) and Ki67 (green) (B). Image at a high magnification of (B) was presented in (C). After EGF, bFGF and LIF withdrawal, NPCs were incubated with FGF8, GDNF and forskolin for 7 days induction and their TH expression increased (E) apparently relative to the control (D). Expression of TH (68 kDa) was confirmed by Western blot analysis (F). Lane F-1, the rat TH as a positive control (60 kDa); F-2, NPCs before induction; F-3, NPCs after mitogen withdrawal and without any cytokines induction for 7 days; F-4, NPCs after mitogen withdrawal and induced by a combination of cytokines (FGF8, GDNF and forskolin) for 7 days. Bar (A) 100 μm; (B) 20 μm; (C) 5 μm; (D and E) 20 μm.

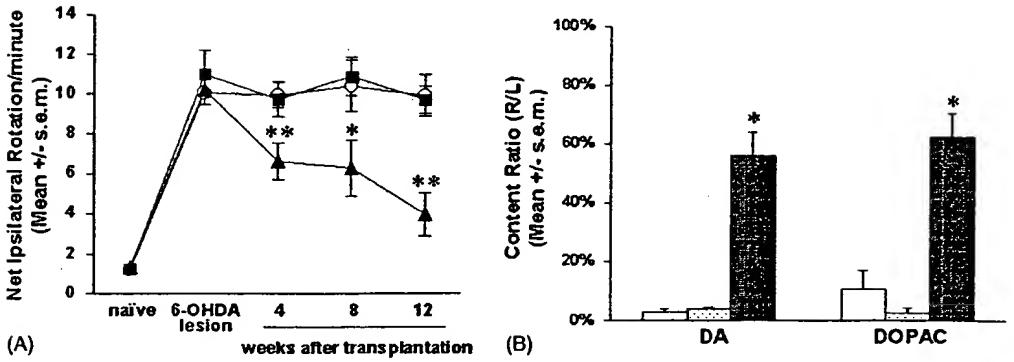

Fig. 2. Behavioral and biochemical analysis of parkinsonian rats. (A) Amphetamine-induced rotation in the group transplanted with pre-differentiated NPCs (▲, $n = 12$) was ameliorated to a greater extent relative to the vehicle control group (○, $n = 12$), as observed from 4 weeks after transplantation and throughout the observation period. This recovery was also significantly different from the intact NPC grafts group (■, $n = 12$). Rats transplanted with intact NPCs showed no significant improvement compared to the control group. (B) DA and DOPAC content in the group transplanted with pre-differentiated NPCs were elevated significantly measured at 12 weeks after transplantation (■, $n = 4$), as compared with the control group (□, $n = 4$). Transplantation of intact NPCs (▨, $n = 4$) did not significantly increase the contents of DA and DOPAC. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.2. Transplantation and behavioral recovery of parkinsonian rats

In the 6-OHDA lesioned side of the brain, most of the DAergic neurons in the substantia nigra and their fibers in the striatum were depleted (data not shown), resulting in an abnormal rat rotation upon amphetamine challenge. In the group transplanted with pre-differentiated NPCs, rats showed significant behavioral recovery relative to vehicle control, which began at 4 weeks after transplantation and sustained behavioral improvement throughout the observing period for up to 12 weeks. The average rotation score decreased from 10.19 ± 0.70 to 6.61 ± 0.93 at 4 weeks, 6.27 ± 1.42 at 8 weeks and 3.93 ± 1.09 at 12 weeks after transplantation. In contrast, transplantation of intact NPCs did not significantly affect behavioral impairment caused by DA lesions (Fig. 2A). The contents of DA and DOPAC in the striatum, which reflect the level of active neurotransmitter and its usage efficiency, were measured by HPLC (Fig. 2B). In order to avoid the individual variances, the result was presented by the ratio of the level of lesioned side versus contralateral (normal) side, which may reflect the actual recovery degree of neurotransmitter. The HPLC result showed that in the group transplanted with pre-differentiated NPCs, the contents of DA and DOPAC were elevated significantly to 56.23 ± 7.61% ($P < 0.05$) and 62.47 ± 7.70% ($P < 0.05$) respectively compared with the negative control group (DA, 3.02 ± 0.84%; DOPAC, 10.95 ± 6.42%), while the intact NPC grafts showed no obvious recovery (DA, 3.39 ± 0.98%; DOPAC, 2.79 ± 1.39%) with this regard.

3.3. Survival, migration and differentiation of transplanted cells

To address the issue of cell survival after transplantation, total human nuclei-positive cells at a series of the 180-μm sections through the graft of each rat brain were counted. The average number for the six rats transplanted with pre-differentiated NPCs was 5024 ± 424 (Fig. 3B), and the number for intact NPCs group was 5752 ± 324 (Fig. 3A; $P > 0.05$ as compared with the group treated with pre-differentiated NPCs). Interestingly, we found that the

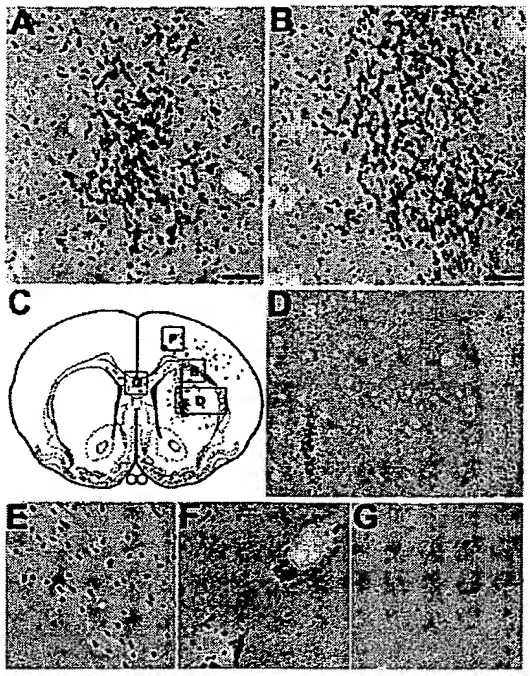

Fig. 3. Survival and migration of engrafted human cells in parkinsonian rats. Engrafted pre-differentiated (B) and intact NPCs (A) survived well in the striatum of parkinsonian rats at the 12 weeks post transplantation and had the similar survival ability tested by human nuclei staining. Two grafts dispersed in the striatum loosely around the needle track, but intact NPCs expressed higher migration potency moving out of the striatum as presented in the schematic drawing (C). They emigrated from the striatum (D), especially into the corpus callosum (E), cortex (F) and even across the middle line into the contralateral corpus callosum (G). Bar (A and B) 50 μm; (D and E) 100 μm; (F) 20 μm; (G) 50 μm.

migration capacity, which is also considered to be one of important properties of NPCs, varied significantly between two grafts. Most of engrafted cells in the rats transplanted with pre-differentiated NPCs were confined to the striatum and loosely scattered around the needle track (Fig. 3B). In contrast, in the group transplanted with intact NPCs, many engrafted cells migrated away from the striatum (see the schematic drawing in Fig. 3C). These cells showed active migration along and within the white matter tracts, mainly to the corpus callosum (Fig. 3E), cortex (Fig. 3F), and even to the contralateral corpus callosum (Fig. 3G). In consistent with previous reports (Ostenfeld et al., 2002), human nuclei-positive cells in the white matter showed an enlongated nucleus parallel to the host fiber tracts. In addition to the migrating on the coronal section, cells underwent a long distance (about 3.4 mm) moving on the sagittal plane along the corpus callosum and cortex, and once the cells emigrated away from the striatum, they did not return into the striatum parenchyma. 66.7% ($n = 6$) of intact NPC grafted brains showed active migration phenomena, whereas, only 16.7% ($n = 6$) of pre-differentiated NPC grafted brains was detected the migration. The average percentage of cells migrating out of the striatum in the all human nuclei-ir survived cells was 27.43 ± 5.19% in the group transplanted with intact NPCs, and 2.12 ± 0.23% transplanted with pre-differentiated NPCs. No human nuclei-positive cells were detected in brains of rats in the negative control group.

Normally, there are little or no DAergic neurons in the striatum. Thus, the TH-ir cells if seen in the striatum were likely derived from the grafted human cells. The result showed that only those rats transplanted with pre-differentiated NPCs exhibited TH-ir cells in their grafted striatum, and this transplantation also resulted in the reinnervation of host striatum (Fig. 4B), which might also contribute to the therapeutic efficacy. However, this was not the case in the NPC-transplanted group (Fig. 4A). Under higher magnification, TH-ir cells with different morphologies were detected around the needle track in the striatum transplanted with pre-differentiated NPCs. Most of them possessed fewer and shorter neurites (Fig. 4C) and some TH-ir cells indeed extended more and longer neurites from the neurosoma (Fig. 4D), exhibiting typical matured DAergic neurons. Grafts of pre-differentiated NPCs yielded the number of 110.67 ± 16.38 TH-ir cells, which was significantly different from the intact NPC grafts ($P < 0.001$). No TH-ir cells were found in the group transplanted with the intact NPCs as well as in the negative control group.

Multipotent differentiation of engrafted cells was detected by double-immunofluorescent staining of human nuclei paired with neurofilament-M for neurons and GFAP for astrocytes, respectively. In the group transplanted with pre-differentiated NPCs, some of the human nuclei-ir cells expressed neurofilament-M (8.02 ± 1.25%) and large numbers expressed GFAP (33.33 ± 7.89%, Fig. 4F). In

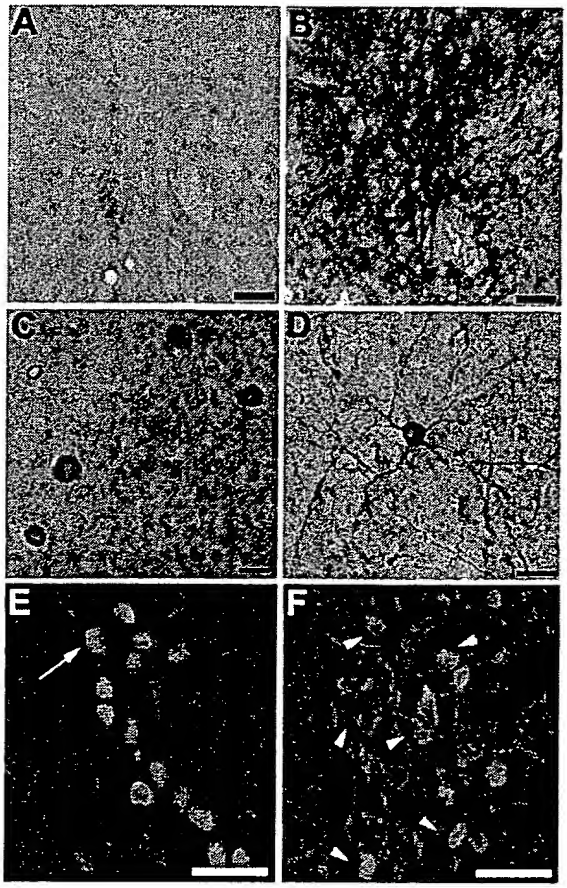

Fig. 4. DAergic and multi-potential differentiation of engrafted cells in parkinsonian rats. TH-ir cells (C and D) and fibres (B) were apparently detected around the needle track in the group transplanted with pre-differentiated NPCs compared with that of intact NPC grafts (A). In the group transplanted with pre-differentiated NPCs, more GFAP-ir astrocytes (arrowhead in F) were observed relative to that of intact NPC grafts (arrow in E). Cells double-stained by anti-human nuclei (green) and GFAP (red) antibodies were considered as those differentiated from grafted human-derived cells detected by CLSM. Bar (A and B) 20 μm; (C) 10 μm; (D–F) 20 μm.

contrast, engrafted intact NPCs were not found to become neuronal phenotype and only a few became astrocytes (2.51 ± 0.70%, Fig. 4E). Thus, our results showed that in vitro pre-differentiated could facilitate the differentiation of NPCs into neural cells after intrastriatal transplantation, especially into GFAP-ir astrocytes.

4. Discussion

Many researches on animal-derived NPCs transplantation therapy for PD have been studied fully and accurately, whereas, the therapeutic efficacy and possible mechanisms in human-derived NPCs therapy were seldom reported. For clinical treatment of PD, an important but as yet unfulfilled goal is the generation of therapeutically large numbers of

DAergic neurons from expanded human cultures, which apparently possesses more advantages than those from animals (Le Belle and Svendsen, 2002). Particularly, the function of mesencephalon-derived human NPCs on PD has not been discussed, possibly due to the difficulties of cultivation and passage of mesencephalic NPCs in vitro compared with those from other brain areas such as hippocampus, SVZ, cortex and so on. Among those in vitro experiments, the previous example of expanded mesencephalon-derived human NPCs being induced to express TH resulted in only modest (approximately 1%) conversion (Storch et al., 2001). Subsequent in vivo investigation indicated that transplantation of intact human NPCs into the striatum of rats resulted primarily in production of glia and GABAergic neurons, not the DAergic neurons needed in the treatment of PD, and even most grafted NPCs still maintained their primary status and did not undergo the differentiation procedure on the earth (Fricker et al., 1999). These findings were among the evidences that suggest that undifferentiated progenitors may not be the appropriate candidate in transplantation therapy of PD. To overcome this problem, some investigations have sought to convert NPCs into the DAergic phenotype prior to grafting (Carvey et al., 2001; Studer et al., 1998) and achieved definite result that the therapeutic efficacy of pre-differentiated rodent NPCs preceded to the intact NPCs. A recent report (Wu et al., 2002) on human NPCs investigation suggested that, for obtaining the high numbers of cells of desired neuronal phenotypes in vivo, priming or induced differentiation of NPCs in vitro prior to grafting is necessary.

In the present study, NPCs generated from ventral mesencephon of aborted human fetus could be expanded well and formed cell clusters of neurospheres in serum-free medium with the addition of EGF, bFGF and LIF in vitro. Induced by a combination of FGF8, GDNF and forskolin for 7 days, NPCs differentiated into TH-ir cells and the conversion ratio was about 20%. FGF8 has been reported involved in the determination of a DAergic neuronal cell fate in vivo and the possible function mechanism is that after binding to its receptor, it activates an intracellular signaling pathway, wherein phosphorylation of MAP kinase leads to an increase in Fos/Jun binding to the AP1 regulatory site on the TH gene (Guo et al., 1998; Mark et al., 1999; Kim et al., 2002). This induction of TH gene expression by this kind of growth factor could be amplified by cooperating with its co-activator forskolin, since it has been well known that as a co-activator, forskolin can up-regulate the level of cyclic AMP, which has been shown to exert critical control of transcription of the TH gene (Hwang et al., 1997; Riaz et al., 2002). GDNF was added into the induction cultures as a neurotrophic factor specific for DAergic neurons (Lin et al., 1993) and expected to promote the differentiation of DAergic neuronal phenotype.

In order to demonstrating that the pre-differentiation is a very key step for transplantation therapy for PD, the differential efficacy of pre-differentiated and intact NPCs was compared and the possible mechanisms were investigated. The present results showed that using an animal model for PD, intrastriatal transplantation of pre-differentiated NPCs derived from human fetal mesencephalon, but not their intact progenitors, ameliorated behavioral deficits in parkinsonian rats. Consistently with this behavioral recovery, DA content in the striatum was also elevated in the group transplanted with pre-differentiated NPCs, providing the basis for the extent of behavioral improvement. One of major metabolites of DA, DOPAC, which reflected the usage efficiency of DA during synaptic transferring procedure, also increased significantly compared with the intact NPC grafts. This result indicated that in the present system of pre-differentiated NPC grafts, DA could be utilized efficiently, suggesting that the behavioral recovery may be benefited from the elevated levels and usage efficiency of neurotransmitter likely produced by pre-differentiated NPC grafts.

What will be the reasons interpreting this differential efficacy between the intact and pre-differentiated human NPCs grafts? The immediate speculations are that transplanted cells did not survive, or, alternatively, they could not differentiate into DAergic neurons after transplantation. Our results demonstrated that the capacities of cell survival of grafted two kinds of NPCs were similar, thus the possibility that different therapeutic effects were a result of different cell survival could be ruled out. Previous works with cell transplantation suggest that behavioral recovery be directly correlated to the number of surviving DAergic neurons in the host striata (Brundin et al., 1998). Adequate number of TH-ir neurons survived in the striata was the basis for animal behavioral improvement. Present result showed that pre-differentiated NPCs generated more functional TH-ir cells in vivo than intact NPCs. This differential DAergic differentiation between the two kinds of grafts implied that pre-differentiation of NPCs prior to transplantation might help lead to the generation of lots of desired phenotype following grafting. Therefore, we were not surprised that transplantation of pre-differentiated human NPCs could induce a behavioral recovery of parkinsonian rats, since more TH-ir cells were detected in this group, which contributed to the differential efficacy of treatment for PD. The surprise, though, is that, given the potential of intact human NPCs to become any type of neurons and the fact that they indeed became DAergic neurons in vitro, transplanted intact NPCs did not give rise to DAergic neurons thereby not improve the behavioral disorder. It should be noted that the differentiation efficiency of grafted intact NPCs was rather low and it generally took about 3 weeks for NPCs to undergo differentiation in vivo (Nishino et al., 2000). Even though, it was still beyond our prediction that, at 12 weeks after transplantation, the engrafted intact NPCs did not differentiated into TH-ir neurons and only a few GFAP-ir astrocytes were sporadically detected in the striatum, suggesting that, in our case, the parkinsonian rats' striatum might not be a suitable environment for human NPCs to spontaneously and sizably differentiate into DAergic neurons. Current finding was in agreement with the previous rodent study (Carvey et al.,

2001) showing that few, if any, human NPCs differentiated into DAergic neurons in the rat striatum in vivo. The failure of grafted NPCs to differentiate into DAergic neurons in the host striatum may be due to the fact that the adult striatum is a non-neurogenic area (Herrera et al., 1999) and that the striatal microenvironment is not favorable for DAergic neuron differentiation.

In addition, the greater migration ability possessed by intact NPCs after transplantation might aggravate the DAergic differentiation in operation site. Compared with pre-differentiated NPCs, intact NPCs showed a greater tendency to emigrate away from the striatum, which resulted in a decreased cell number in the target site, including those with potential to differentiate into DAergic neurons. Similar observations were also found by other studies showing active migration of NPCs after being transplanted into the adult rat brain (Caldwell et al., 2001; Ostenfeld et al., 2002; Fricker et al., 1999; Englund et al., 2002). Present study indicated that pre-differentiation by the cytokine mixture of FGF8, GDNF and forskolin in vitro down-regulated the extensive migration of grafted NPCs in vivo. What needs to conduct next step may be to find out the molecular mechanisms regulating the migration capability of NPCs.

As described above, we used anti-human nuclei antibody to detect survived human cells in vivo and demonstrated that at 12 weeks after transplantation, both types of engrafted cells, i.e., the pre-differentiated and the intact human NPCs, have similar survival abilities in the brains of parkinsonian rats. However, it is important to note that the conversion rate of DAergic phenotype was very limited even in the group transplanted with pre-differentiated NPCs. This reduced TH-ir cells number occurred even though a significantly greater number of DAergic neurons could be obtained in the in vitro induction experiment prior to transplantation. We had confused with the fact that behavioral impairment alleviated by pre-differentiated NPC grafts was to a greater extent than the number of TH-ir cells detected in the striatum. In the present study, a large number of grafted astrocytes were seen within the striatum along with the transplantion of pre-differentiated NPCs. This may indicate some important roles played by these exogenous astrocytes in improving local striatal environments and thus the overall behavioral recovery after transplantation. One of roles that these astrocytes might play was to synthesize and secret certain neurotrophic factors that could rescue and promote striatal DAergic fibers to regenerate or restore the injured tissues, resulting in ultimate behavioral improvements in parkinsonian rats (Knusel et al., 1991; Kordower et al., 1999). We did not know what factors suppressed the expression of TH-ir phenotype exactly. It seemed that immunoresponse was not included in the reasons (Brundin et al., 1985) because an immunosuppressive agent, cyclosporin A, was administrated throughout the experimental period and obvious immunorejection phenomena was not observed in this study up to 12 weeks after transplantation. Probably boosting particular neurotrophic factors with the pre-differentiated NPC grafts or transplanting genetic modified NPCs may protect grafted neurons from oxidative and metabolic stress, and provide epigenetic trophic support (Dawson and Dawson, 2002).

In conclusion, our results demonstrated that intrastriatal transplantation of pre-differentiated human mesencephalic NPCs, but not intact NPCs, ameliorated behavioral impairments of parkinsonian rats. This difference in the therapeutic effects may result from the fact that intact NPCs emigrated actively away from the transplanted site and were not able to differentiate into DAergic neurons in vivo, while pre-differentiated NPCs gave rise to more DAergic neurons and astrocytes. Contrary to intact NPCs, pre-differentiation in vitro is a very key step to improve the disordered behaviors of parkinsonian animal models after transplantation. Our study may provide a practical protocol for the treatment of PD through neural cells transplantation.

Acknowledgements

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